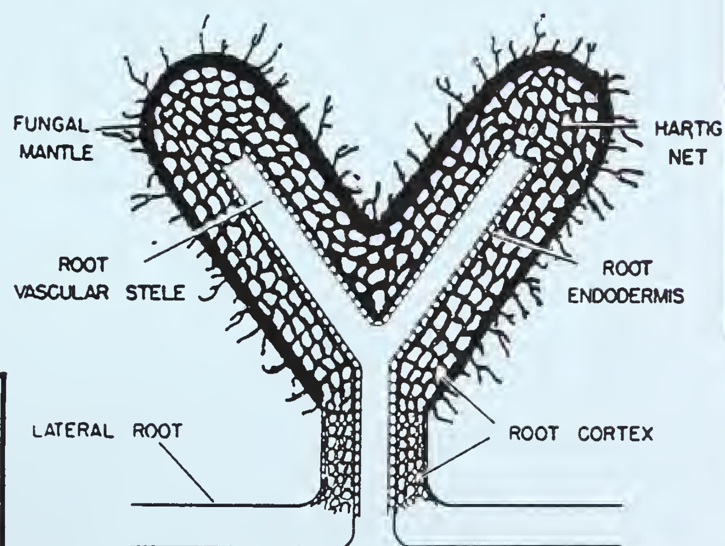
Mycorrhizal Inoculation of Container Grown Ponderosa Pine Seedlings



FOREST PEST MANAGEMENT
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MYCORRHIZAL INOCULATION OF CONTAINER GROWN PONDERSOA PINE SEEDLINGS

By

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INTRODUCTION

There has been considerable interest in recent years in the culturing of mycorrhizae on forest tree seedlings. Recent studies have shown that nursery stock can be inoculated with mycorrhizal fungi and that the resultant seedlings are larger and better suited for outplanting than nonmycorrhizal seedlings.

Mycorrhizal fungi are known to be an important component of the forest ecosystem. Many tree species, especially members of the pine family, are obligate mycorrhiza formers; they required these fungi for survival. There are three major types of mycorrhizae; ectomycorrhizae, which will be represented by the acronym ECM in this manuscript, are the principal kind found on coniferous trees.

ECM benefit their tree hosts in many ways. Trees with good ECM development have a larger root surface area for water and nutrient absorption. These fungi are able to increase the absorptive capacity of their host for plant nutrients, especially phosphorus, which are normally not available to the tree. ECM are physiologically active for a longer period of time compared to uninfected roots. Research has shown that ECM can increase resistance to pathogenic root fungi such as *Pythium* and *Phytophthora*. From the forester's standpoint, the most practical benefit of ECM is the increased tolerance of seedlings to adverse site conditions, including extremes of soil pH, high soil temperatures and toxic substances.

Ponderosa pine (*Pinus ponderosa* Laws.) forms ECM with many species of fungi in the natural forest environment. Most tree seedlings are naturally inoculated with indigenous species of ECM fungi either in the nursery bed or soon after outplanting on forested sites. The advent of the containerized tree seedling with its sterilized substrate and special cultural treatments restricts good ECM formation during the greenhouse rotation. It is important that container stock be inoculated with proper ECM fungi, especially if the tubelings are to be outplanted on currently nonforested sites.

Pisolithus tinctorius (pers.) Coker and Couch is an ECM fungus that is a known associate of ponderosa pine and is distributed world-wide. Seedlings with this type of ECM grow larger and have higher survival rates than those without the benefits of these fungal associations. Whether *Pisolithus* ECM can aid in reforestation and aforestation of central Rocky Mountain forests needs to be tested.

ECM inoculation of containerized stock requires a nominal expenditure which can easily be justified in view of the manifold benefits. Based on current figures, inoculation with ECM fungi will require an additional cost of 20% which could easily be offset by an estimated yearly savings of $4,500 for each 1% increase in plantation survival. If the additional costs of site preparation and replanting were considered, these savings would be even greater. It is apparent that the benefit-cost ratio is highly favorable for ECM inoculations, especially concerning adverse sites where reforestation is a special problem.

OBJECTIVES

1. Determine the practicality of artificially inoculating container-grown ponderosa pine seedlings with the ECM fungus *Pisolithus tinctorius*.

2. Determine the most effective rate of inoculum mix with potting soil to produce satisfactory ECM development.

METHODS AND PROCEDURES

The type of ECM inoculum used in this study was vegetative inoculum consisting of specially treated potting soil containing fungal mycelia. This inoculum was a mixture of vermiculite and peat moss which was aseptically inoculated with *P. tinctorius* and incubated in large glass jars until the fungus completely colonized the substrate. Due to the exacting requirements of producing large quantities of this inoculum, 100 liters were purchased on a contract basis from Dr. C. P. P. Reid of the Department of Forest and Wood Sciencies at Colorado State University.

The inoculum preparation consisted of removing the vegetative inoculum from the incubation jars and thoroughly washing it to remove excess nutrient solution which was used to grow the fungus. The inoculum was then screened through a 5 mm wire mesh to break up aggregated particles and assure effective mixing with the regular potting soil.

A range of inoculum mixing rates were tried to determine the most efficient ratio with the regular vermiculite-peat moss potting soil. Treatments consisted of inoculum application rates of 0%, 5%, 7.5%, 15% and 25% by volume with the inoculum mixed into the potting soil prior to seeding. Inoculum and potting soil were mixed in a portable cement mixer. An additional treatment consisted of mixing pine duff from an established pine stand into the potting soil, an inoculation technique frequently used in the past.

After each treatment the potting mix was distributed into Leach Jumbo containers which are 10 cubic cm. in volume. The individual container cells were filled 3/4 full, seeded with 3 ponderosa pine seeds per cell, and the seeds covered with a layer of perlite. This procedure yielded approximately 500 replications per treatment. The seeded containers were assembled into racks and placed on the same bench of the greenhouse. Following seedling emergence, all the containers were thinned to one tree per cell. All seedlings were allowed to develop under the standard environmental regime for pines at the Colorado State Forest Service greenhouse at Fort Collins, Colorado.

Seedling growth and pattern of ECM development were monitored at 3-month intervals during the 9-month greenhouse period. During these evaluations 10-20 seedlings from each treatment were removed from their containers and the root plug examined for ECM development. In addition to this non-destructive visual rating, 2 typical seedlings from each treatment were randomly selected and taken to the Forest Insect and Disease Management Laboratory in Denver for destructive analysis. These seedlings were measured for shoot height, root length, stem caliper and shoot-root ratio on a dry weight basis. ECM development was evaluated on the basis of color, texture, and morphology of the ECM and percent coverage of ECM on the seedling root system. The presence of ECM was confirmed through microscopic examination of stained freezing microtome sections; the existence of a fungal mantle and Hartig net were distinguishing criteria (Figure 1). Fungal cultures were made on Hagem's agar to attempt reisolation of the ECM fungus on artificial media.

A final measurement of ECM inoculation success was made at the end of the 9-month greenhouse period. Seedlings from all Pisolithus treatments were rated for ECM and microtome sections and cultures used for confirmation of the visual appraisal. ECM establishment was rated as percent coverage of the seedling root system; only seedlings with ECM over 50% of the short roots on the root wad exterior were called mycorrhizal. Seedlings with lesser amounts of ECM were eliminated from consideration and only seedlings with no obvious ECM were considered nonmycorrhizal. Shoot height and stem caliper were measured on 50 mycorrhizal seedlings and 50 nonmycorrhizal seedlings to determine if any size differences could be detected.

Soil samples were assayed for Pythium spp. and Fusarium spp. at the regular evaluation times using the diagnostic procedure of David Johnson (pers. comm.). Pythium was isolated on water agar and a selective Pythium medium. Counts of Fusarium propagules were made on Nash and Snyder's Peptone PCNB medium using 0.0025 g. air-dry soil per replication.

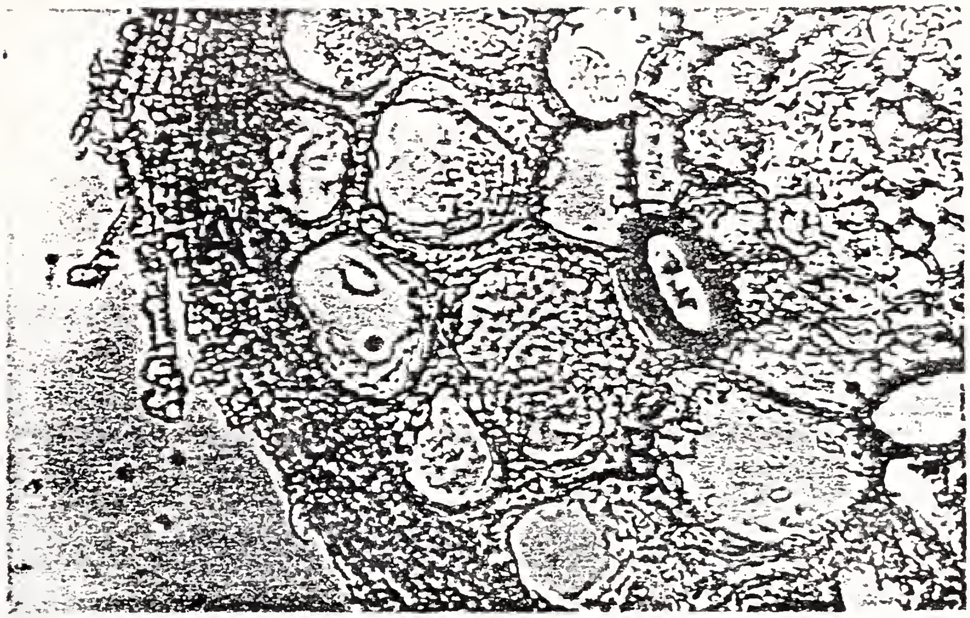


Figure 1 - Photomicrograph of an ectomycorrhiza of Pisolithus tinctorius in cross-section, showing the distinguishing characteristics: A - The fungal mantle and B - The Hartig net (Approximately 1000X)

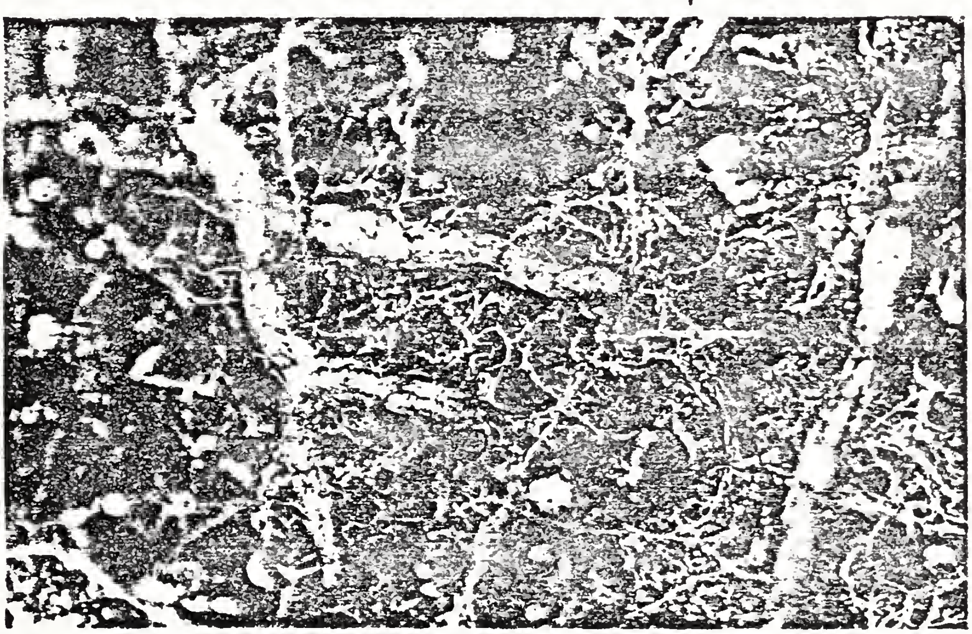


Figure 4 - A typical ectomycorrhiza of Pisolithus tinctorius on the surface of the seedling container root plug. Note extension of fungal hyphae between mycorrhizae and into the potting soil. (Approximately 10X)

RESULTS AND DISCUSSION

Seedlings suffer early losses from damping-off

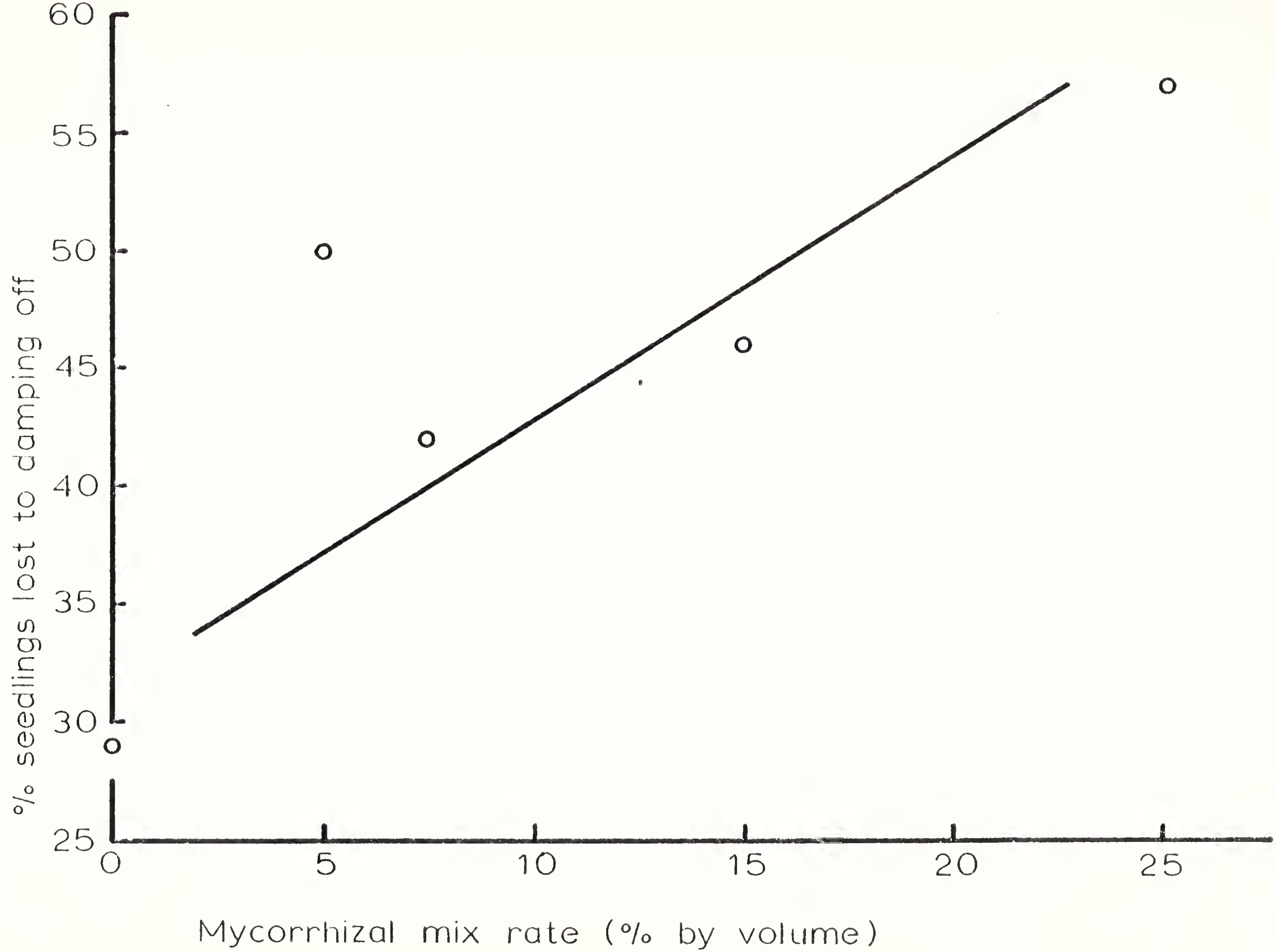
Serious damping-off losses were apparent soon after seedling emergence; in some cases over 50% of the seedlings either did not emerge or wilted soon after emergence. Although damping-off fungi are always present to some degree in nurseries, losses of this magnitude indicate significant pathogen population levels. Fungal cultures from diseased seedlings and potting soil revealed that Pythium spp. were involved; seedling signs and symptoms were also characteristic of this pathogen.

An inventory of seedling losses in the various treatments indicated a strong relationship between inoculum mix rate and percent damping-off (Figure 2). This probably resulted from insufficient washing of the Pisolithus inoculum because the nutrient solution used to grow the ECM fungus will also stimulate pathogenic fungi.

However, the fact that almost 30% of the seedlings in the 0% treatment were lost to damping-off indicates that an unusually high population level of damping-off fungi must have been present (Figure 2). There are several sources for damping-off fungi in a greenhouse environment: the potting mix, the seed or the irrigation system. Due to the fact that losses were not noted in other seedlings in the same greenhouse, the irrigation water was probably not at fault. Seeds from the same source were cultured for pathogenic fungi, but none were found. The vermiculite and peat moss potting soil appears to be the prime suspect, especially because it had not been sterilized prior to sowing. Steam or methyl bromide sterilization would eliminate these pathogens as well as any saprophytic bacteria, fungi or nematodes that could compete with the CM fungus in the potting mixture.

Even though empty container cells were reseeded to compensate for thse losses, this effort was only partially successful because damping-off losses were evident for 6 months after the original sowing date. Fungal cultures taken from potting soil and symptomatic seedlings at the 3-month and 6-month sampling dates revealed that a succession of pathogenic fungi had occurred. Whereas the original losses were caused by Pythium, the later cultures were negative for Pythium but showed high levels of Fusarium spp., another serious cause of damping-off. Characteristic Fusarium symptoms were apparent on symptomatic seedlings. This fungal succession has been documented in other studies and reflects the type of injury typical of these fungi. Pythium is a pre-emergence or immediate post-emergence pathogen because it favors the succulent young tissue of the emergence seedling. Fusarium is responsible for post-emergence damping-off and a vascular wilt until the seedling begins secondary thickening of the hypocotyl. One species of this genus can cause a root rot of older seedlings.

FIGURE 2 -- Damping-off losses were related to amount of mycorrhizal inoculum added to the potting mix.



Fungal isolations from a variety of seedling containers provided data to determine a relationship between the population level of Fusarium and the condition of the host (Figure 3). Container cells in which seedlings never emerged had the highest fungal population and even containers with apparently healthy trees had some level of Fusarium. Seedlings that had emerged and died and stunted seedlings were intermediate in their Fusarium populations. These data indicate that Fusarium is common in the greenhouse environment but that some critical population level or predisposing environmental condition may be necessary before significant damage occurs.

Ectomycorrhizal establishment and development

Evaluation of seedling root systems during the 3-month and 6-month examinations revealed poor ECM development in all treatments. A small proportion of the roots in the higher _P. tinctorius_ applications exhibited rudimentary ECM which were dichotomously branched with few external hyphae. Lack of good ECM development at this stage of seedling growth was interpreted as inoculation failure because other investigators have reported satisfactory ECM development at 4-6 months.

Additional seedlings were sampled at 7 months to attempt to determine reasons for the inoculation failure. Upon removal of the tubelings from their containers, numerous well-developed ECM could be seen on many of the seedling root plugs. Microscopic examination confirmed that these were typical ECM of _P. tinctorius_: primarily dichotomously branched, yellow-brown in color with numerous hyphal strands extending between individual ECM and outward into the potting mixture (Figure 4). These ECM were most common in seedlings of the 15% and 25% mix treatments. Eighty percent of the isolations made from these ECM were positive for _P. tinctorius_, confirming the presence of this fungus.

The final 9-month evaluation confirmed that success of ECM establishment varied with inoculum application rate (Table 1). ECM development varied almost linearly with amount of inoculum in the treatment: the 25% and 15% mix rates were most effective, the 7.5% was intermediate and the 5% treatment did not differ from the pine duff and 0% treatments. At the higher application rates approximately one-third of the pine seedlings exhibited some _P. tinctorius_ ECM development. Considering the initial damping-off problems and long period of ECM inactivity, this level of success is significant. An application rate of 20% inoculum by volume (average of 15% and 25% rate) to potting soil appears to be an effective rate for future inoculations.

A small percentage of roots in the pine duff and 0% inoculum treatments developed ECM (Table 1), although they were different from the _Pisolithus_ type. Two types of ECM were found on the pine duff seedlings: one was

FIGURE 3 -- A relationship was observed between damping-off losses caused by Fusarium spp. and the health of the ponderosa pine seedlings.

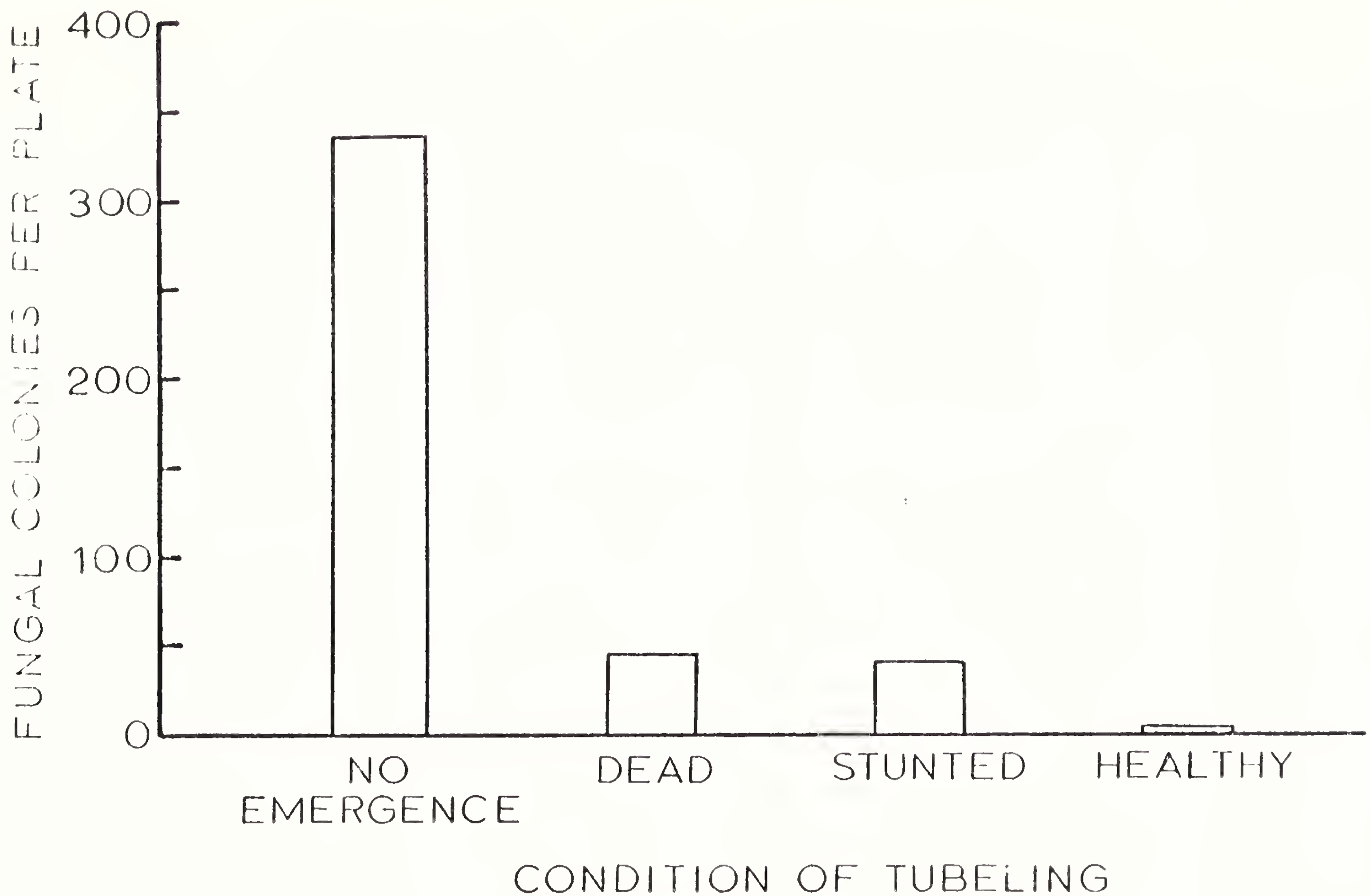


TABLE 1 -- Success of ectomyocrrhizal inoculations and various application rates after a 9-month greenhouse period. 1/

Inoculation Treatment	Degree of ectomycorrhizal (ECM) establishment > 50% ECM	<50% ECM	No ECM
Pisolithus tinctorius			
25% Mix	20%	14%	66%
15% Mix	15%	14%	71%
7.5% Mix	3%	15%	82%
5% Mix	1%	5%	94%
Pine Duff	1%	7%	92%
0% Mix	0%	3%	97%

1/ Based on examination of 100 seedlings per treatment

dark brown to black, primarily dichotomous with pure white surface mycelia and numerous hyphae emanating from them whereas the second type was cream-colored, monopodial to slightly bifurcate with few external hyphae. In general, however, the pine duff additive was not an effective means of inoculating container seedlings. The fact that some seedlings in the 0% inoculum treatment had some ECM verifies that accidental inoculation with airborne spores or infected potting soil is possible but not common enough to provide a reliable source of inoculum in greenhouse environments.

Comparison of shoot height and stem caliper between mycorrhizal (> 50% ECM) and nonmycorrhizal seedlings (no ECM) demonstrates that pine seedlings with good ECM development are significantly larger than seedlings without the benefit of these fungal associations. Average shoot height was 10.8 cm. for mycorrhizal seedlings compared with 7.5 cm. for nonmycorrhizal seedlings and the stem caliper measurements for the two groups were 2.5 mm. and 2.0 mm. respectively. These differences were significant at the 0.001 level based on a paired t-test with 49 df. This growth stimulation has been noted in many other ECM inoculation experiments; in some cases, ECM seedlings were twice as large as the controls. These larger ECM seedlings may be the result of the direct benefits of ECM or indirect effects such as disease protection.

The 7-month lag period between inoculation and ECM development suggests that P. tinctorius is capable of existing outside the ECM state for considerable time periods. Whether Pisolithus was maintained as a saprophyte or persisted in a dormant state is not known.

The sudden proliferation of Pisolithus ECM subsequent to this 7-month period of inactivity suggests that some inhibitory factor was operative. The seedling hardening-off period was initiated just prior to this 7-month evaluation and the appearance of ECM. During this period fertilization and irrigation schedules were reduced, CO_2 production was terminated, photoperiod was shortened, and soil and air temperatures were allowed to equilibrate with the outside winter environment. Of these factors, fertilization and irrigation are the most suspect as they have been implicated as inhibitory to ECM in other experiments. Heavy fertilization, especially nitrogen, is known to be detrimental to good ECM development. Overwatering may also inhibit ECM because P. tinctorius is known to be strongly aerobic and constant saturation prohibits good soil aeration. This hypothesis is supported by observations of hypertrophied lenticels on many seedling root systems and the abundance of Pisolithus ECM on the exterior of the container plug where aeration is better.

CONCLUSIONS AND RECOMMENDATIONS

Application of vegetative inoculum to standard potting mix is an effective means of inoculating ECM into container-grown ponderosa pine seedlings. Inoculation with *Pisolithus tinctorius* resulted in ECM formation on seedling root systems during a 9-month greenhouse tenure. A mix rate of 20% inoculum by volume appeared to be the most effective of the different application rates. Application of pine duff as an inoculum was not an effective means of stimulating ECM on container stock. Significant levels of ECM did not develop on uninoculated seedlings as a result of airborne spores or other accidental inoculations.

Heavy damping-off losses, up to 50% in certain treatments, occurred soon after seedling emergence. Percent of damping-off loss increased with higher rates of *P. tinctorius* inoculum, although a high population level of these fungi was present even in the noninoculated seedlings. In future experiments the inoculum should be thoroughly washed and leached of culture nutrients which can stimulate damping-off fungi. It is speculated that these damping-off fungi were introduced on unsterilzed potting soil.

Both *Pythium* spp. and *Fusarium* spp. were isolated from diseased seedlings; Pythium caused greatest loss early in the rotation whereas Fusarium continued to be a problem until the seedlings began secondary thickening. Fusarium population levels were related to the condition of the condition of the host seedling. Although all seedlings showed some level of Fusarium, it appears that a certain population threshold or predisposing environmental conditions were necessary before significant damage occurred.

Few ECM were found on the inoculated seedlings during the first 7 months growth. A sudden proliferation of *P. tinctorius* ECM following this 7-month period of inactivity suggests that this fungus is capable of existing in a saprophytic or dormant state for extended periods of time.

Lack of ECM development during this 7-month period suggests that some factor in the nursery environment was inhibiting this process. High levels of fertilizer and heavy irrigation may have been inhibitory to ECM development. Additional studies on the effects of these factors are needed to develop application schedules which are optimal for both seedling growth and ECM development.

Comparison of dimensional measurements of ECM seedlings and nonmycorrhizal seedlings confirms that *Pisolithus* ECM are beneficial to pine seedling growth. Theoretically, this size advantage should be reflected in increased survival and growth rate of outplanted seedlings, especially on nonforested sites.

Suggestions for improvement of inoculation technique

1. Applying the inoculum as a upper stratum to container cells may be more effective than mixing it into the potting soil because soil aeration and fertilizer levels should be more conducive to ECM development at the top of the cell. The ECM-infected potting mix could be added to partially filled container cells and the seeds sown on top of this layer. When conditions become favorable, the ECM fungus should grow rapidly downward, infecting roots lower in the container. This technique will also insure that the same quantity of inoculum is delivered to each cell whereas large differences in mix rate can result when the inoculum is mixed with the regular potting soil.

2. Changes in the potting mixture will improve the soil environment for ECM development. Soil structure will be helped by incorporating more vermiculite and less peat moss into the soil mixture; vermiculite is coarser in texture which should encourage aeration.

3. Damping-off losses could be reduced by preventing pathogenic contamination. Potting soils should be sterilized with steam or methyl bromide to eliminate fungal pathogens and destroy any other organisms that could inhibit the growth of the desired ECM fungus. Seed could be treated with a surface sterilant such as hydrogen peroxide to destroy any potential pathogens on the seed coat. Fungicide treatments are not recommended because of possible phytotoxic side effects to the tree seedling.

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